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Article

Biogenic silver nanoparticles from Iris tuberosa as potential preservative in cosmetic products

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Abstract: Biogenic-Silver nanoparticles emerge as new nanosilver platfmorm that allow to obtain silver nanoparticles via "green chemistry". In our study we obtained biogenic-Silver nanoparticles from *Iris tuberosa* leaves extract. Nanoparticles were characterized by UV-vis spectroscopy, dinnamical light scattering technique and Transmission electron microscope, showing spheric and irregular nanoparticles with 5 to 50 nm in diameter. Antioxidant and antimicrobial properties were evaluated against typical microbial contaminants found in cosmetic products, showing high antioxidant and antimicrobial properties. We formulated a natural moisturizing cream with biogenic-silver nanoparticles to evaluate the preservative efficiency trought challenge test, confirming that our nanoparticles are a promising alternative to use as cosmetic preservative.

Keywords: nanotechnology; silver nanoparticles; antimicrobial; biotechnology; phitochemistry; plant extract; green chemistry; cosmetics; preservatives.

1. Introduction

The Global cosmetic market is projected to register a CAGR (Compound annual growth rate) of 4.3% during forecast period (2016-2022) and anticipated to reach $429.8 billion by 2022 [1]. The rising market need to put special interest in multidimensional control to oversee toxic ingredients and microbial contamination [2]. The contamination of cosmetics products (CPs) is one of the most problematic risk for consumer's health. During the period between 2008 and 2014 Rapid Alert System (RAPEX) of the European Commission (EC), notified 62 cases of CPs contaminated by microorganism and were recalled due to these incidences [2].

In general terms, the most usually alterations in CPs is through microorganism contamination or the result of over exposition to atmospheric oxygen [3]. Every time a new CP is opened the microorganisms that are present in the atmosphere reach the product. However, those products that have the appropriate conservatives avoid the development of these microorganisms, and allow the product to remain safe. Usually, the origin of contamination in new CPs comes from one or more sources, such as raw material, environment, equipment used during the manufacture, primary packaging material, or personnel handling the product. When microorganisms come into contact with a cosmetic product, and it has insufficient preservation, the product can be affected in different ways that include the appearance of mold on the product, separation of phases of the emulsions, loss of viscosity, change of the aroma, rancidity of fats [2]. Although the risk of developing disease from CPs is very low, the bacteria present in these products can cause irritations or infections, especially if the product comes into contact with broken skin. About 2 to 4% of dermatological consultations are due to dermatitis caused by cosmetics, however ad-

verse reactions affect not only the skin in form of irritation or peeling but also as conjunctivitis, asthma, urticaria, angioedema or pneumonia [4].To avoid these problems, substances or additives are included in the cosmetic industry in the formulation of products that provide stability to the product. There are different forms of conservation that can range from physical, radiation and chemical conservation [5].

The preservative systems most used in cosmetics are natural antioxidants (Vitamin E) and synthetic (sodium bisulfite) as well as antimicrobials and antifungals such as parahydroxybenzoates (Parabens) or formaldehyde releasing agents. An effective preservative should have a broad activity spectrum and a longer duration than the cosmetic product itself, being equivalent to the expected shelf-life plus the usage time [6].

Some cosmetic preservatives such as Parabens, Triclosan, Benzalkonium chloride, Imidazolidinyl urea and Diazolidinyl urea have shown different adverse effects in humans such as DNA damage [7], antiandrogenic activity [8], estrogenicity [9], endocrine disruptors [10] [11], cytotoxic and genotoxic effects on humans lymphocytes, risk of cancer in humans, allergic reactions , reproductive disorders and also environmental and animal toxicity [1] [11] [12] [13]. For these reasons, some of the traditional preservative systems are currently being questioned, so new molecules with biocidal activity or molecules that create an unfavorable environment for microorganisms are being sought.

Nanotechnology and its "Nanomaterials" (NMs) arises as new and promising alternative to traditional and hazardous ingredients in CPs due to their unique physicochemical properties [14]. In the EU, the official definition of NMs in cosmetic is given as: "an insoluble or bio-persistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm". There are a lot of types of NMs which are currently used in CPs and most of them are collected in different regulatory organizations, in EU, the main regulatory framework for cosmetic products is the EC Regulation 1223/2009 which have a list of all NMs used in CPs [15]. The last updated catalogue consisted of 29 NMs.

Silver nanoparticles (AgNPs) are a promising NMs used in different areas such as medical, food , health care, consumer and industrial purposes, due to their unique physical and chemical properties, especially for its behavior as a biocide [16]. Whitin the NMs most used in cosmetic colloidal silver (nano) or (AgNPs) is widely used as chemopreventive agent in sunscreams and preservative agent in CPs and its consider as safety preservative by Scientifyc Committee on Consumer Safety (SCCS) [17].

There are mainly three approaches to obtain AgNPs: chemical, physical and biological methods [18]. Biological methods are a new ecological alternative which does not employ toxic or non-enviromental friendly reagents in its synthesis [19]. This approach utilizes natural resources such as plant extracts, bacteria, fungi and other templates (DNA, viruses) as a source of reducing agents for the generation of AgNPs when they react with silver molecules in different conditions [20] [21].

There are different biogenic silver nanoparticles available, and made from several plant extracts such as green tea, *Salvia lerifolia, Psidium guayaba , Nebumbo nucifera* and *Emblica officinalis* among many others. These nanoparticles have been tested and used successfully as biocide agents [22].

Iris tuberosa mainly known as snake´s head, widow iris, black iris or velvet flower-de-luce, is a species of non-rhizomatous plant belonging to Iridaceae family. *Iris tuberosa* has been used in traditional medicine for acne treatment, for skin care and drandruff treatment.

the aim of this work is to formulate a new biogenic-AgNPs as preservative in moisturizing cream. The water leaves extract of *Iris tuberosa* was used as precursor of reducing agents to obtain bio-AgNPs. The AgNPs were characterized and evaluated using UV-Vis spectrum, Dynamic Light Scattering (DLS) technique and Transmission Electron Microscope (TEM, furthermore, the antimicrobial and antioxidant properties were carried out, and finally a moisturizing cream incorporating our biogeninc-AgNps was formulate and its efficacy and efficiency was evaluated as preservative agents through challenge test.

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2. Results and Discussion

2.1. Polyphenols and flavonoids contents in *Iris tuberosa* extract (ITE)

Polyphenols and flavonoids are one of the most commonly used agents to produce AgNPs by green chemistry trough plant extracts [22] [23]. These compounds are used as reducing agents to produce silver nanoparticles reacting with a silver precursor. Many silver nanoparticles have been successfully obtained using plant extracts especially with those with high content of polyphenols and flavonoids. In this study, aqueous extract obtained from *Iris tuberosa* (ITE) was obtained and total content of polyphenols and flavonoids was quantified using Folin-Ciocalteau method, and colorimetric method with $AlCl_3*6H_2O$, respectively. The obtained results showed that the ITE extract contains an amount of total polyphenols of 1305 mg of GAE/100 gr of dried weight, and a total flavonoid content of 1966 mg of QE/100 gr of dried weight. Other extracts used as precursors of silver nanoparticles contain these compounds, for example aqueous extract of pomegranate leaves, where AgNPs were obtained has a total polyphenols of 397 mg GAE/gr and a total flavonoids, of 126 RE/gr [23].

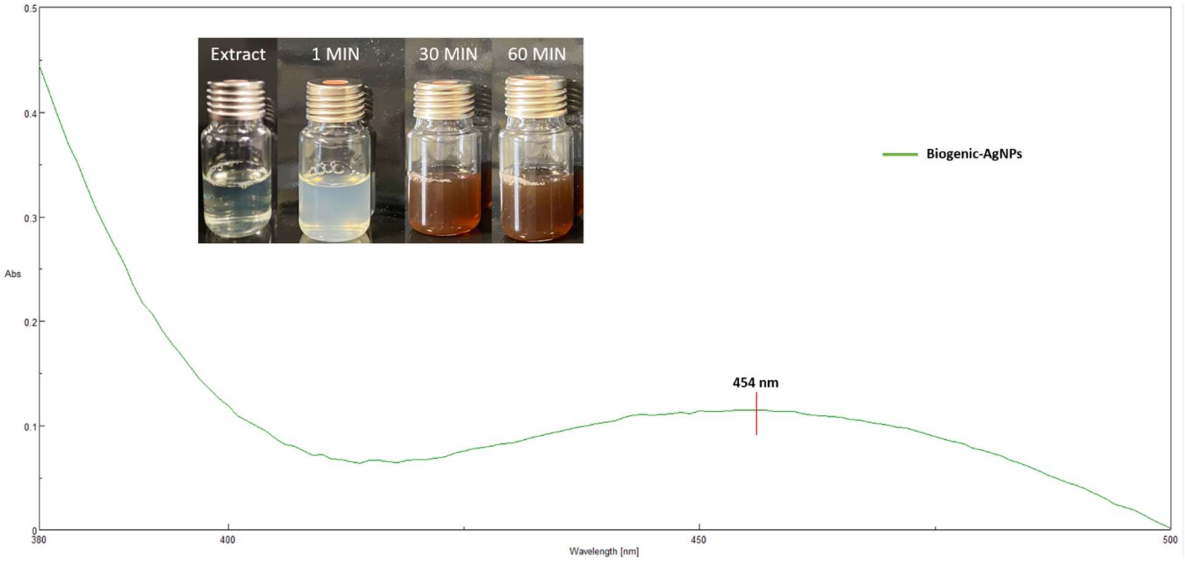

Figure 1. UV-spectrum of *Iris tuberosa* extract and Biogenic-AgNPs. Inset it is shown the change in color of silver nanoparticle formation.

2.2 Synthesis and characterization of biogenic-AgNPs

2.2.1 Synthesis and UV-vis characterization of biogenic-AgNP .

Silver nanoparticles were biosynthesized using ITE aqueous extract. As soon as the $AgNO_3$ solution was added to leaves aqueous extract from ITE under vigorous stirring, immediately the silver nitrate solution color turns from light yellow to brownish yellow, indicating the formation of silver nanoparticles as depicted in Figure 1. The brownish yellow color is due to the excitation of free electrons in the nanoparticles and occurs in 60 min at room temperature and under white light. After 24 hours, no further color change was detected due to stabilization of the synthesized nanoparticles.

To evaluate the biosynthesized nanoparticles of AgNPs through green synthesis using the aqueous ITE extract, UV-visible scan was done from 380 to 500 nm, to confirm the

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presence of nanoparticles in the sample based on their optical absorbance peaks. The absorption spectra of the synthesized silver nanoparticles was recorded against water. The reduction of silver ions was confirmed by UV-vis spectra, where the maximum absorbance was seen at 454 nm (Figure 1). The characteristic peak of silver nanoparticle known as the surface plasmon resonance timely increased and is in the range 420–480 nm, as shown in silver nanoparticles obtained from *Lysiloma acapulcensis* [24], and with a *Althernantera sessilis* extract, where similar peaks were obtained [25]. ITE extract acts as a reductant agent mediating the synthesis, as well as stabilization, of the silver nanoparticles with characteristic plasmon resonance band in their characteristic range.

2.2.2 DLS measures of biogenic-AgNPs.

The hydrodynamic diameters of AgNPs in aqueous solution were determined using Dynamical light scattering. DLS or photonic correlation spectroscopy is a spectroscopy technique that allow the evaluation of nanoparticles size and surface charge of stern layer trough Brownian movement [26]. The main advantage of DLS is the short time required to perform the measurement, and the relatively low cost of the apparatus, therefore, DLS become the preferred method for nanoparticle sizing however, DLS method has several drawbacks with respect to the influence of dust particles or small amounts of large aggregates. The results listed in Table 1 and Figure 2c, show the nanoscale of biogenic-AgNPs was 116 nm with Polydispersity index (PDI) of 0.3, due to a range of nanoparticles between 24 nm to 105 nm. The higher sizes detected are the consequence of aggregation of smaller nanoparticles, which lead to a high value of PDI. Most literature indicate a similar particle size of AgNPs ranging from 10-800nm as described in neem, onion and tomato extracts with a PDI between 0.2-1.0 [27]. Similar results were obtained using a *Berberis* leaf extract where the silver nanoparticles showed a size between 90-100 nm, and a PDI of 0.3, the authors of the study point out that although these nanoparticles had a high DL values, aggregation of small nanoparticles were detected by TEM analysis [28].

Zeta potential determination allow the estimation of the surface charge, which can be used for cheking the physical stability of nanoparticles [29]. A large positive or negative value of zeta potential of nanoparticles point out good physical stability of nanoparticles, due to electrostatic repulsion of individual particles. Biogenic-AgNPs from ITE showed in Table 1 and Figure 2d, a Z-value of -27.5 mV indicating a stable and monodisperse suspensions [30]. A zeta potential value other than −30 mV to +30 mV is generally considered to have sufficient repulsive force to attain better physical colloidal stability. A small zeta potential value can result in particle aggregation and flocculation due to the Van der Waals attractive forces act upon them.

Table 1. DLS measures of Biogenic-AgNPs

Formulation	Average Size (nm)[b]	PDI	Z-value (mV)
Biogenic-AgNPs	116.4±4.10	0.3±0.02	-27.5±0.83

2.2.3 TEM images of biogenic-AgNPs.

To gain more information on the obtained nanoparticles, their size and morphology were analysed by transmission electron microscope (TEM). TEM micrographs shown in Figure 2a and b, revealed spherical and irregular shape. Micrograph shows a nanoparticles with a maximum size of 50 nm and a minimum approximately of 5 nm, which was in concordance with the data obtained in DLS measurement, and indicated that the high value is due to aggregation of small nanoparticles. Smaller-sized of Ag nanoparticles have

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many positive characteristics, such as chemical stability, good conductivity, cancer treatment, antibacterial activities, antifungal, antiviral and biofilm eradication which would make them attractive for many industrial applications.

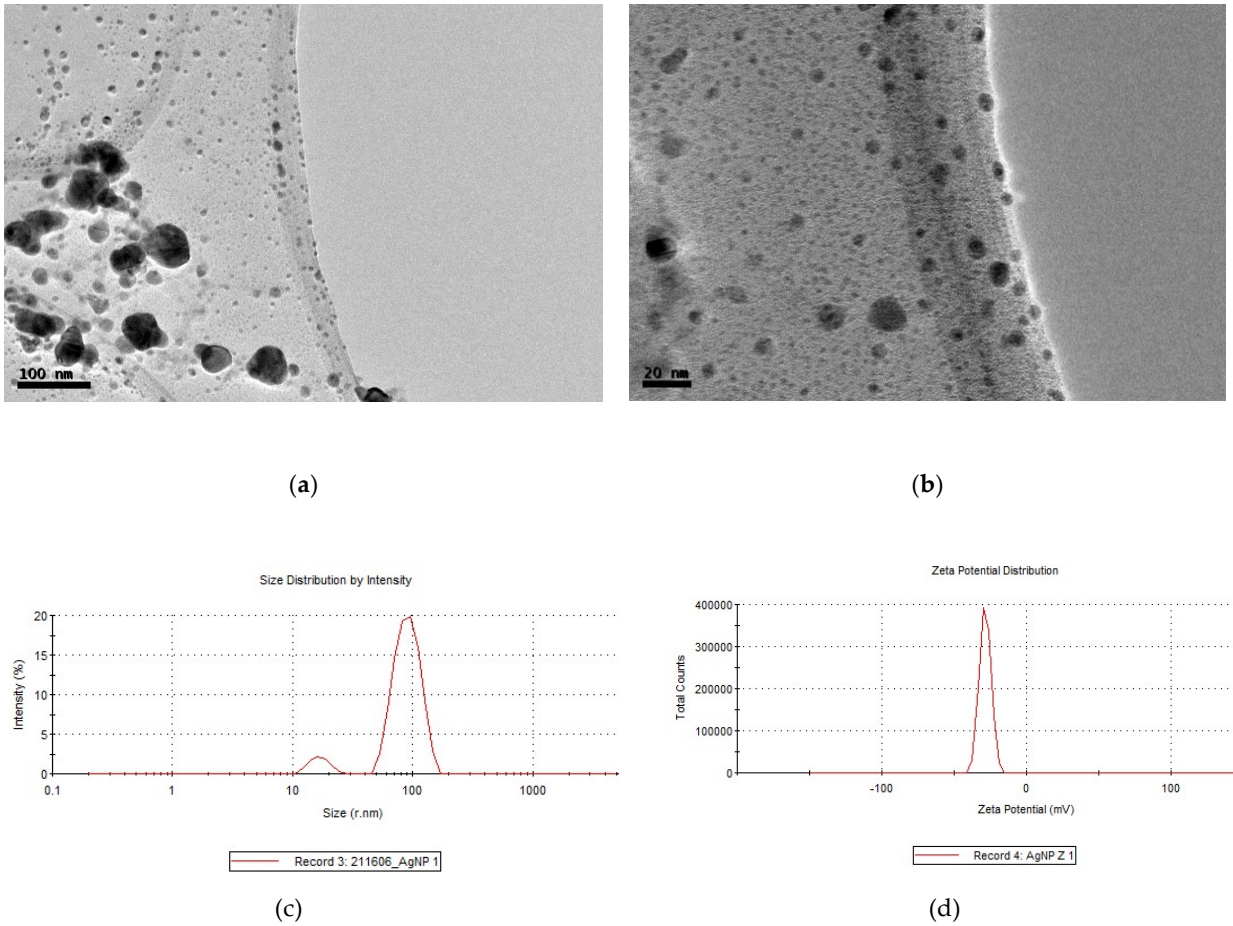

Figure 2. Nanoparticles characterization by microscopy and Z potential. a) and b) TEM micrographs, c) Size distribution, d) Z potential of Biogenic-AgNPs

2.3 Antimicrobial activity of biogenic-AgNPs

It is generally assumed that the attachment of AgNPs onto the cell wall and membrane, the damages of intracellular caused by AgNPs and silver ions, lead to oxidative stress. Augmentation of the concentration of reactive oxygen species (ROS) can be attributed to a higher rate of formation, or to a disruption in the scavenging pathways, these mechanisms either singly or concurrently are involved in the antibacterial actions and conduct a cellular inactivation.

In vitro evaluation of antimicrobial properties of biogenic silver nanoparticles against typical pathogens contaminants in cosmetics, *Escherochia. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida. albicans* and *Aspergillus brasiliensis* was realized by broth microdilution method. Data from Table 2 indicate that the AgNP has a high MIC ranging from 14.1 ug/ml against *P. aeruginosa*, *S. aureus*, and *A. brasiliensis* to 66.7 ug/ml in *C. albicans*, while the control MIC was lower ranging between 185 ug/ml against *C. albicans* and *A. brasiliensis* and 3333 ug/ml in *S. aureus*. Silver nanoparticles are considered the most widely explored antibacterial nanoagent due to their broad-spectrum antimicrobial properties and robust antimicrobial effectiveness against bacteria, viruses, and fungi. AgNP

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acts as antimicrobial agent mainly trough three mechanisms: I) cell wall and membrane damage, inhibiting the respiratory process through interaction between silver and compounds of thiol groups [28] , II) intracellular penetration and production of DNA and protein damage [31] and III) oxidative stress through ROS, producing membrane breakage and increasing membrane permeability, which finally resulted in disruption of electron transport chain and leakage of the cellular content [22]. Table 3 shows similar MICs between gram – and gram + species revealing that the antimicrobial activity is unrelated to the class of bacteria. However, other AgNPs synthesized by *Berberis vulgaris* extract showed high affinity for gram negative species as *E. coli* than gram positive species as *S. aureus* [28].

Biogenic-AgNPs shows a lower MICs agains *S.Aureginosa* and *S.Aureus* than *E.coli* with 14.1 μg/ml and 44.4 μg/ml respectively. Other reports using silver nanoparticles from *Alpia katsumadai* showed MICs of 20 μg/mL against *S. aureus* and *E. coli* and 40 μg/ml against *P. aureginosa* [32]. More effective inhibition was obtained by silver nanoparticles from *Lotus* extract showing a MICs against *P. aureginosa* and *S. aureus* of 10 μg/ml [33]. Aragão et al showed a MICs for *E. coli* and *S. aureus* of 34.3 and 81.2 μg/ml, respectively with silver nanoparticles made from *Gracilaria birdiae*. These results confirm that our biogenic-AgNPs have a good antibacterial activity similar to thos reported in other studies. *C.Albicans* was less sensitive to Biogenic-AgNPs with a MICs of 66.7 while *A.Brasiliensis* has a MIC of 14.1 μg/ml, exhibiting reasonably strong antifungal activity (Table 2). Such results are in a good agreement with the previously published paper considering silver nanoparticles from *Enteromorpha flexuosa* and *A. brasiliensis* showing MICs against *C. albicans* ranging between 25 and 300 μg/mL, repectively [34] [35].

Microorganism	Control MIC (μg/mL)	Biogenic-AgNP MIC (μg/mL)
E. coli	1111.0	44.4
P. aeruginosa	1111.0	14.1
S. aureus	3333.0	14.1
C. albicans	185.0	66.7
A. brasiliensis	185.0	14.1

Table 3. Minimum inhibitory concentrations (μg/ml) of control drugs (Gentamicin and Tebuconazole) and Biogenic-AgNPs

2.4 Antioxidant properties of biogenic-AgNPs

Nowadays, there is a great interest regarding products with antioxidants activity. This products are frequently used as food additives and cosmetics and are regulated by law, being of special interest food antioxidants in the prevention of inflammatory lesions, nutritional deficiencies, autoimmune diseases, Parkinson's, heart attacks. myocardium, aging, neoplasms, neurodegeneration, atherosclerosis, and diabetes. The antioxidant activity of a compound is due to the capture of free radicals and ROS, produced by cellular metabolism or in response to external factors that are inactivated, avoiding or preventing degenerative diseases in humans caused by oxidation of nucleic acids, proteins, or lipids. To check the possible antioxidant activity of this nanoparticles, and expand their uses, we evaluated these properties by the DPPH scavenging method. DPPH was chosen for its simplicity, it is one of the few organic radicals with nitrogen atoms in its structure, which

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confers the stability as a result of the delocalization of an unpaired electron on the molecule. This delocalization also causes an intensification of the purplish color characteristic of the radical, which in a metallonic medium absorbs at 515 nm. This purplish coloration of the solution is attenuated in the presence of an antioxidant that can donate or transfer a hydrogen atom, giving a yellowish color due to the reduced form of DPHP-H. [36]. Biogenic-AgNPs exhibited a DPPH radical scavenging reduction (%) from 24-65% at maximum dose of 1.4 mg/ml as depicted in Figure 3. It can be therefore concluded that the biogenic-AgNPs formed displayed a strong antioxidant activity similar to silver nanoparticles formulated from *Alternathera sessilis* having a maximum of DPPH scavenging reduction of 62 % [25].

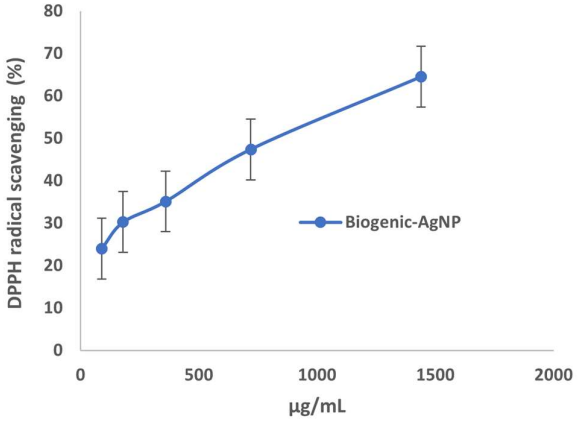

Figure 3. DPPH scavenging reduction of Biogenic-AgNPs

Table 4. Cream composition. A (Aqueous phase), B (Oil phase), C (thermolabile compounds)

Ingredient	Control-Cream (%)	AgNP-Cream (%)
Water (A)	56.3	57.3
Vegetable Glycerin (A)	4.2	4.2
Macadamia ternifolia seed oil (B)	12.5	12.5
Orbingnya oleifera seed oil (B)	10	10
Helianthus annus seed oil (B)	5	5
Montanov 68 (B)	5	5
Argania spinosa kernel oil (B)	2	2
Biosaccharide gum-1 ©	2	2
Malva silvestris flower extract ©	1.27	1.27
Vitamin E (C)	0.5	0.5
Parfum (C)	0.2	0.2
Benzyl alcohol (C)	0.91	-
Ethylhexylglycerin (C)	0.12	-
AgNPs (C)	-	0.007

2.5 Preservative efficacy of biogenic-AgNPs into formulated cream

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The cosmetic industry was one of the first to use and develop patents applications using nanotechnology and nanomaterials. Applications cover product formulation, packaging as well as cosmetic manufacturing equipment. In cosmetic products, nanomaterials are used as active carrier substances and / or as formulation support in order to improve the effectiveness of the product. In order to evaluate the use of biogenic-AgNPs as a preservative agent a cream was formulated as dispayed in Table 4.

Following the UNE-EN ISO 11930, control and AgNPs based creams were inoculated separtely with *E. coli*, *P. aeruginosa*, *S. aureus*, *C. albicans* and *A. brasiliensis* to evaluate the preservative efficacy through challenge test. After 2, 7, 14 and 28 days one gram of both creams was spread by drigalski spatula in petri dishes containing a PDA for fungi and LB for bacteria to count CFUs. The results of challenge test are shown in Table 5. The UNE-EN ISO 11930 established two types of acceptance criteria. A) For bacteria, the preservative should reduce the number of bacteria to 2 logs on day 2 , 3 logs on day 7 and no CFU day 28; for fungi, the policy dictates a reduction of 2 logs on day 2 and no CFU on day 28; B) For bacteria and fungi, the preservative should inhibit the growth to 3 logs and 1 logs on day 14, respectively and no CFU on day 28 in both microorganism. Based on these criteria and the results showed in Table 5, our biogenic-AgNPs are an effective preservative system satisfying the A and B criteria of acceptance of mentioned normative. The control cream was formulated using benzyl alcohol as a mild preservative agent, which obtained by sugars fermentation and it is commonly used in natural cosmetics due to its safety profile [37], while and ethylhexylglycerin is a multifunctional agent commonly used as natural preservative and skin conditioning agent [38]. The cream control showed less preservative effect than Biogenic AgNP cream. Thus, our proposed formulation confirm that the biogenic-AgNPs is more effective preservative to cosmetics than common " eco-friendly" preservatives.

Microorganism	Control-Cream		AgNP-Cream	
P. aureginosa	T=0	1.5E+05	T=0	1.5E+05
Count CFU/gr	T=2	1.5E+05	T=2	0
	T=7	1.5E+05	T=7	0
	T=14	1.5E+05	T=14	0
	T=28	1.2E+04	T=28	0
E. coli	T=0	1.5E+05	T=0	1.5E+05
Count CFU/gr	T=2	1.5E+05	T=2	0
	T=7	1.5E+05	T=7	0
	T=14	1.5E+05	T=14	0
	T=28	4.8E+05	T=28	0
S. aureus	T=0	1.5E+05	T=0	1.5E+05
Count CFU/gr	T=2	1.1E+05	T=2	5.3E+01
	T=7	8.6E+02	T=7	0
	T=14	3.8E+03	T=14	0
	T=28	4.4E+03	T=28	0
A. brasiliensis	T=0	1.5E+05	T=0	1.5E+05
Count CFU/gr	T=7	1.0E+02	T=7	6.7E+01
	T=14	0	T=14	0
	T=28	0	T=28	0
C. albicans	T=0	1.4E+0.4	T=0	1.4E+0.4
Count CFU/gr	T=7	1.9E+03	T=7	0
	T=14	2.5E+03	T=14	0
	T=28	1.8E+03	T=28	0

Table 5. Challenge test in formulated Control-Cream and AgNP-Cream

4. Materials and Methods

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All the reagents were purchased from Sigma Aldrich (Spain). Plants of *Irirs Tuberosa* were collected in Botanic Garden of Castilla-La Mancha. Microoganism were purchased from American Type Culture Collection *E. coli* (ATCC25922), *P.a aureginosa* (ATCC27853), *S. aureus* (ATCC 6538), *C. albicans* (ATCC 10231), *A. brasiliensis* (ATCC16404).

4.1 Plant extract preparation

Iris tuberosa leaves were pulverized and lyophilized. Then, one gram of lyophilized was added to 100 ml of milliQ water and kept in a heated plate for 15 minutes at 80ºC. The aqueous extract was filtered through 0.45 µM millipore filter and stored at -20ºC.

4.2 Content of *Flavonoids and Polyphenols in Iris tuberosa extract*

Determination of total polyphenols. The total amount of polyphenols in the aqueous extract were performance through Folin-Ciocalteau method [39]. Briefly, 0.1 ml of aqueous extract were mixed with 2 ml of 2% Na_2CO_3, 2.8 ml of H_2O and 0.1 ml of Folin-Ciocalteau reagent. After mixing, the color change was measured by the absorbance at 750 nm. The calibration curve was done with Gallic acid as standard at different concentrations (10-200 ppm). The experiment was realized in triplicate.

Determination of total flavonoid content. The total amount of Flavonoids in the aqueous extract was carried out through a colorimetric method with $AlCl_3*6H_2O$ [39]. Briefly, 0.5 ml of aqueous extract were mixed with, 1.5 ml of Ethanol, 0.1 ml of $AlCl_3*6H_2O$ at 10%, 0.1 ml of CH_3COOK 1 M and 2.8 ml of H_2O. After mixing, the color change was measured at 415 nm. The calibration curve was realized with quercetin as standard at different concentrations (8-500 ppm). The experiment was realized in triplicate.

4.3 Synthesis of biogenic-Silver nanoparticles

Synthesis of AgNPs. Following a method previously described [24], with sevral modifications, 10 ml of $AgNO_3$ 25mM were added dropwise at a caudal of 2 ml/min into 10 ml of *I. tuberosa* aquose extract under vigorous stirring. The suspension was kept in agitation under white light, monitoring color change of the suspension during time. The AgNPs were collected after centrifugation at 15000 rpm for 15 minutes at 4 ºC, and washed several times with milliQ water and freeze dried at -40ºC.

4.4 Nanoparticles characterization.

UV-vis spectra analysis. The AgNPs formation was performed by measuring the UV-vis spectrum of the reaction mixure against *I. tuberosa* extract as a blank. The spectral analysis was done using a double bean PerkinElmer spectrophotometer at a resolution of 1 nm from 380 nm to 500 nm.

Particle size measurements. The average sizes, polydispersities and Z-potentials of the AgNPs were measured using a Zetasizer Nano ZS (Malvern Instruments). Data were analyzed using the multimodal number distribution software included in the instrument. All measures were done in triplicate.

Morphology studies of AgNPs. High resolution electron microscope images were ontained on a Jeol JEM 210 TEM microscope operating at 200 kV and equipped with an Oxford Link EDS detector. The resulting images were analyzed using Digital Micrograph™ software from Gatan.

4.5 Antimicrobial assay.

The antimicrobial activity and minimum inhibitory concentration (MIC) of biogenic-AgNPs were tested against the most commonly contaminants in cosmetics, and those that UNI EN ISO 11930:2012 recomend for preservative efficacy evaluation. Challenge test using *P. aeruginosa, E. coli, S.s aureus, A. brasiliensis* and *C. albicans* was carried out using the broth microdilution method [40]. Stock cultures were prepared from Culti-Loops ™ (Sigma-Aldrich) in Nutrient broth (NB) and Potato dextrose Broth (PDB) at 37 °C for 24 °C. Standarized inoculum was then created by dilution in Müller Hinton medium to a final density of 0.5 McFarland units by densitometer McFarland type DEN-1B (Biosan, Riga, Latvia). AgNPs were tested in concentrations of 133 µg/ml to 0.5 µg/ml. Gentamicin

(for bacteria) and Tebuconazole (for mold and yeast) were used as standards. After treatment, plates were incubated 24 hours at 37 °C for bacteria and 48 hours at 30 °C for yeast and fungi.

4.6 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

FRS, free radical scavenging activity was determined as described previously [41]0.5 ml of each concentration (1.5 mg/ml, 750 µg/ml, 375 µg/ml, 187.5 µg/ml, and 93.8 µg/ml) of synthetized AgNPs were mixed with 0.2 mM methanolic DPPH radical solution (0.5 ml). Equal volume (1 ml) of AgNPs and DPPH solution (0.2 mM in ethanol) were mixed and kept in the dark at room temperature for 30 min. The absorbance of the solution was measured at 517 nm. The FRS was calculated by %= (A0-A1/A0) x 100.

4.7 Moisturizing cream formulation

Two types of moisturizing creams were formulated. Control Cream with standard preservatives (Benzyl alcohol + Ethylhexylglycerin) and AgNP-Cream preservative-free with the addition of biogenic-AgNPs. The composition of both creams is shown in Table 4. Oil phase (B) and Water phase (A) were preheated at 70 °C to achieve the fusion of oils and waxes present in this phase. Then, B was added slowly under agitation in homogenizer to form an oil/water emulsion. When the cream was cooled the thermolabile compounds (C), preservatives in the case of control cream and biogenic-AgNPs, at dose of 70 µg/ml (Maximum of MIC value), in the AgNP-cream were added to cooled cream under continuous agitation.

4.8 Preservative activity of AgNPs in formulated cream

The preservative efficacy of AgNPs in moisturizing cream was carried out following the criteria of UNE-EN ISO 11930. Twenty grams of each cream (Control and AgNP) was diluted with sterile NaCl solution at 0.9 %. Then, each cream was inoculated with 10^5 UFC/ml for bacteria, 10^4 CFU/ml for yeast and 10^3 CFU/ml for mold. Contaminated creams were stored at room temperature for 30 days. After 2, 7, 14 and 28 days one gram of each contaminated cream was diluted and spread in Petri dishes to count CFUs of bacteria. Yeast and mold contaminations were evaluated at 7, 14, and 28 days using the same method.

5. Conclusions

The incorporation of preservatives in cosmetic formulations is necessary, because these products are source of nutrients for bacteria, fungi and yeasts. However, finding the right type of preservative or preservative system to incorporate into each formula, which satisfies all preservation and toxicological safety criteria, represents a challenge for the cosmetic microbiologist. Our Biogenic AgNPs preservative showed a broad spectrum of antimicrobial activity, has a known chemical structure, is completely soluble in water, is compatible with all the ingredients of the formulation, and it is cheap to produce. Therefore can be easily translate to the cosmetic industry**.**

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